AD_____

Award Number: W81XWH-14-1-0012

TITLE: Development of a Diagnostic Tool to Detect DNA Methylation Biomarkers for Early-Stage Lung Cancer

PRINCIPAL INVESTIGATOR: Chongli Yuan

CONTRACTING ORGANIZATION: Purdue University
West Lafayette, IN 47907

REPORT DATE: February 2015

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
February 2015	Final	15 Nov 2013 - 14 Nov 2014

4. TITLE AND SUBTITLE

Development of a Diagnostic Tool to Detect DNA Methylation Biomarkers for Early-Stage Lung Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0012

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Chongli Yuan, Julie Liu, Seong-Eun Kim, Charng-yun Lin, Agnes Mendonca, Renay Su

E-Mail: cyuan@purdue.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

480 Stadium Mall Dr.,
Purdue University
West Lafayette, IN 47907

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Biomarkers based on DNA methylation patterns combine genetic (inherited) and epigenetic (environmental) manifestations of lung-cancer risk and have great promise in detecting early-stage lung cancer. The current approaches for finding novel methylation targets, however, either do not provide DNA base-pair resolution or are laborious to perform.

This application overcomes these challenges by combining protein engineering and fluorescence spectroscopy to develop a highly sensitive molecular complex for mapping DNA methylation patterns. We have successfully produced the proposed molecular probes. The performance of the DNA methylation-recognition domain and our detection platform has been successfully validated. The developed probe is ready to be transitioned to mapping DNA methylation patterns within a gene locus. This technology will facilitate clinical screening for early-stage lung cancer patients.

15. SUBJECT TERMS
Lung cancer, DNA methylation and protein sensor

16. SECURITY CLASSIFICATION OF: U			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified		USAMRMC

INTRODUCTION: Biomarkers based on DNA methylation patterns combine genetic (inherited) and epigenetic (environmental) manifestations of lung-cancer risk and have great promise in detecting early-stage lung cancer. However, the current approaches for finding novel methylation targets either do not provide DNA base-pair resolution or are laborious to perform. This application overcomes these challenges by combining protein engineering and fluorescence spectroscopy to develop a highly sensitive molecular complex for mapping DNA methylation patterns. We aim to 1) engineer molecular probes to detect methylated DNA and 2) identify DNA methylation at high spatial resolution.

KEYWORDS: DNA methylation, lung cancer, protein sensor.

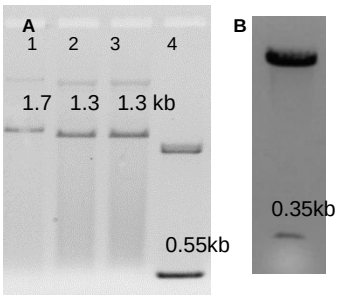

Fig. 1: (A) DNA gel showing the cloning plasmids with the right insert sizes. Lane 1: A DNA recognition domain that binds to 17bp DNA. Lane 2-3: DNA recognition domains that bind to 13bp DNA. Lane 4: the cloning vector containing the second piece of probe. (B) The cloning plasmid of the DNA recognition domain fused to other functional components. All inserts show the expected molecular weight.

ACCOMPLISHMENTS:

What were the major goals of the project?

There are two specific aims of this project. Specific Aim1: Engineer molecular probes to detect spatial patterns of methylated DNA. (Accomplished in December, 2014). Specific Aim 2: Identify DNA methylation with high spatial resolution (on-going progress, percentage of completion is 50%)

What was accomplished under these goals?

Specific Aim1: Engineer molecular probes to detect spatial patterns of methylated DNA.

Task 1: Clone the gene sequences for the molecular probes. The proposed probe contains two pieces. The **first** piece will include: 1) a *DNA recognition* domain that recognizes the specific DNA sequence of interest and 2) one half of the *leucine zipper* pair. The **second** piece will include 1) the second half of the *leucine zipper* pair, 2) a *flexible linker* flanked by a FRET pair that determines the local (within 30 bp) methylation pattern, and 3) an *MBD domain* that specifically binds methylated DNA. The DNA recognition domains and the second pieces of the probe were successfully cloned by March, 2014 as shown in Fig.1A. The addition of the zipper domain to the first piece, however, takes us extra time to complete because the selected DNA recognition domain contains highly-repetitive sequences and is not compatible with regular PCR. We screened a large number of DNA polymerase and finally identified a DNA polymerase strain (Prime Star DNA polymerase) from Clontech that enables the successful cloning of the fusion protein by Oct., 2014. A DNA gel showing the successfully cloned fusion protein was included in Fig. 1B. All clones are verified by DNA sequencing.

Task 1.2: Produce purified molecular probes. We started the production of both pieces of protein probes immediately after the cloning product was confirmed by DNA sequencing. Both pieces of probes were expressed in large excess in *E.coli* strains as shown in Fig. 2.

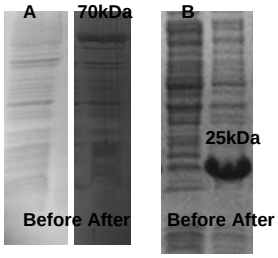

Fig. 2: SDS-PAGE of extracts from whole cells transformed with the cloning vectors of (A) the first- and (B) the second- piece of our probe, respectively, before and after induction. The molecular weights of the overexpressed bands are in close proximity with the expected molecular weight of the proteins.

Task 1.3: Investigate binding affinity. Both pieces of probes contains a 6 x Histidine tag on their C-termini and were purified using Nickel-agarose beads following the standard procedure.The binding affinity of both pieces of probes was examined using DNA band-shift assays. Both probes demonstrate the expected preference for selected DNA sequences and DNA methylation as shown in Fig.3. The binding affinity of the DNA-recognition domain is significantly higher than that of the methyl-binding domain. The probe binding is therefore going to be primarily driven by the DNA sequence recognition domain.

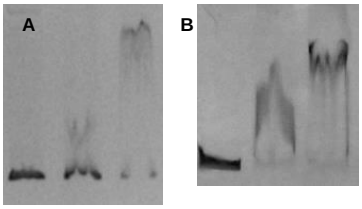

Fig. 3: DNA binding gel showing (A) the DNA recognition domain binds specifically to the target DNA sequences (the protein to DNA ratio from left to right was 0, 4, and 12); and (B) the MBD domain binds specifically to methylated DNA fragments (the protein to DNA ratio was 0, 200 and 340 from left to right).

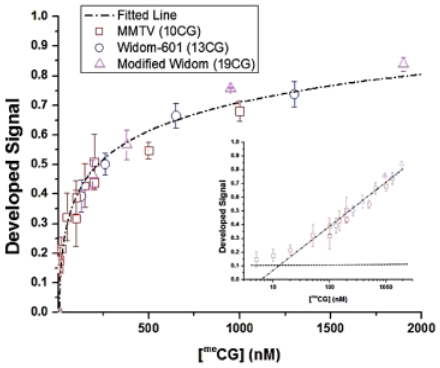

Fig. 4: Developed fluorescence signals as a function of [^{me}CG] measured using 18 different DNA samples.

Task 1.4: Determine minimum detection limit. Because of the unexpected delay in preparing the DNA recognition fragment, we proceeded to characterize the detection limit of MBD-piece using fluorescence spectroscopy. Our results suggest that our methyl-probes can sensitively detect DNA methylation (^{me}CG) concentrations ranging from 20 nM - 2 µM as shown in Fig.4. We expect comparable detection limit when combining both pieces of probes.

Specific Aim2: Identify DNA methylation with high spatial resolution.

Task 2.1 Design a library of DNA constructs in which the location of the single methylated CpG site is varied. We have successfully designed a library of DNA fragments consisting of varying DNA sequence. The CG site was designed to be 1-2 bp away from each other to determine the resolution of our probes. All DNA fragments are methylated using bacterial methyltransferase. Since only a single CG sites is incorporated in these DNA sequence, we verified the methylation level of these DNA fragments and found that they are all close to 100%

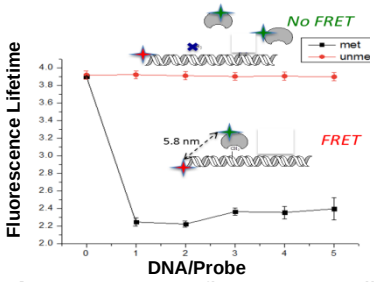

Fig. 5: Average fluorescence lifetime of MBD-probes mixed with DNA of different concentrations. The DNA substrate containing one CG sites which is 5.8nm away from the end of the DNA.

Task 2.2 Determine the region in which the molecular probe detects methylation. Due to the unexpected delay in the production of the second piece of probes, we did not manage to complete this proposed task on a timely basis. Alternatively, we performed FRET experiments using our designed DNA library by incorporating a fluorescent acceptor molecule on the 5' end of DNA. The MBD-probe was tagged with a donor molecule. The collected FRET agrees well with the expected theoretical value, confirming the feasibility of our proposed approach as shown in Fig. 5.

Task 2.3 Determine the spatial resolution of the molecular probes. Similar to task 2.2, this task was also affected by the delay in production of the DNA recognition pieces. Alternatively, we experimentally tested the feasibility of adapting our proposed experiments to single-molecule platform. We successfully tested the feasibility of immobilizing selected DNA fragments on solid substrates and observed the dynamic binding and unbinding behavior of the MBD-piece of probes to methylated DNA fragments as shown in Fig.6.

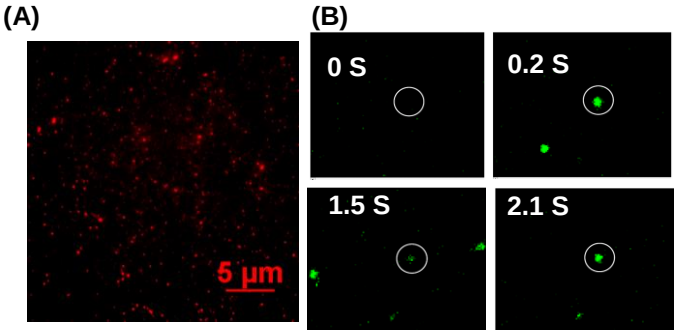

Fig. 6: (A) Single-molecule image of 57 bp DNA fragments conjugated to coverslip. (B) Single-molecule image of fluorescently-tagged methyl-binding domain incubated with surface-conjugated DNA. The time-lapse images show the dynamic binding and unbinding of MBD1 to the DNA. 1 pixel = 60 nm.

What opportunities for training and professional development has the project provided?

Training opportunities: this grant has supported four graduate students and has provided them interdisciplinary training on protein engineering, biophysics and epigenetics.

How were the results disseminated to communities of interest?

Dissemination of results: This proposal has already resulted in one journal publication (Kim, SE, Chang, M. and Yuan, C., one-pot approach for examining the DNA methylation patterns using an engineered methyl-probe, Biosensor and Bioelectronics, 58:333 (2014)) and one conference

talk (Yuan, C., Development of molecular sensors to monitor cellular epigenetic features, Symposium on Chemical and Biotechnology Engineering 2014, Singapore). Two more publications are being planned in the near future.

The PIs have also made contacts with lung cancer oncologist (Dr. Hanna Nasser) at Medical School, Indiana University to discuss about the future applications of our probes in clinical applications.

What do you plan to do during the next reporting period to accomplish the goals?

If extra funding is available to support the continuation of this project, we will have validated both pieces of probes and demonstrate their usage using both solution and single-molecule FRET experiments.

IMPACT:

What was the impact on the development of the principal discipline of the project?

This proposal aims to design and demonstrate proof-of-concept for these molecular probes. We have successfully completed the production of both pieces of these molecular probes and have validated the usage of the methyl-binding domains for quantifying DNA methylation levels. Once we have completed the validation studies combining both pieces of probes, the next steps are to demonstrate the use of the probe with a large number of clinical samples and to find new modification patterns that indicate the development of lung cancer.

What was the impact on other disciplines?

The developed probes can be used to reveal methylation patterns *in situ* and can be of broad interest to the chromatin biology community.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

The survival rate of lung cancer patients is largely dependent on the stage at which it is detected; diagnosis at an early stage is affiliated with a high recovery rate. The current screening approach, i.e., low-dosage CT, however, has a high false-positive rate. A biomarker that indicates early-stage lung cancer and can be obtained in a non-invasive manner using molecular probes as we developed in this study has great potential in improving the accuracy of current lung-cancer screening approaches and consequently increase the survival rates of patients with lung cancer.

CHANGES/PROBLEMS:

Changes in approach and reasons for change.

No significant changes were made to our approach.

Actual or anticipated problems or delays and actions or plans to resolve them.

The major issue that we have with this project is the production of the DNA-recognition domain. After exploring many alternative cloning strategies, we finally succeeded in producing the fusion protein. The feasibility of the detection platform has been verified, meanwhile, primarily using the MBD-probe part. Currently, our probe pair is ready to be validated in FRET assays.

Changes that had a significant impact on expenditures.

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazard, and/or select agent.

Nothing to report.

PRODUCTS

Publications, conference papers, and presentations

Journal Publications: This proposal has already resulted in one journal publication (Kim, SE, Chang, M. and Yuan, C., one-pot approach for examining the DNA methylation patterns using an engineered methyl-probe, Biosensor and Bioelectronics, 58:333 (2014)). Two more publications are being planned in the near future.

Books or other non-periodical, one-time publication: nothing to report.

Other publications, conference papers, and presentation: This proposal has resulted in one conference talk (Yuan, C., Development of molecular sensors to monitor cellular epigenetic features, Symposium on Chemical and Biotechnology Engineering 2014, Singapore).

Website(s) or other interest site(s)

Nothing to report.

Technologies or techniques.

The detailed sequence of all protein and DNA constructs will be made available in the resulting publications. These constructs will also be made available to those requesting them for research purposes. Purdue University agreed to the Uniform Biological Materials Transfer Agreement (UBMTA), and any transfers of materials will be subject to the standard Purdue University Material Transfer Agreement.

Inventions, patent applications, and/or license.

Nothing to report.

Other products.

Nothing to report.

PARTICIPANTS & OTHER COLLABORATIN ORGANIZATIONS

What individuals have worked on the project?

Name	Chongli Yuan	Julie Liu	Seong-Eun Kim	Agnes Mendonca	Charng-yu Lin	Renay Su
Project Role	PI	Co-PI	Graduate student	Graduate student	Graduate student	Graduate student
Nearest person month worked	2 months	1 months	11 months	4 months	5 months	5 months
Contribution to project	Dr. Yuan oversees the entire project and work with graduate students to design all experiments.	Dr. Liu is primarily in charge of the cloning and expression of protein probes.	Ms. Kim is primarily in charge of charactering the performance of the DNA methylation recognition domain	Ms. Mendonca is primarily in charge of characterizing the performance of DNA-recognition domain.	Mr. Lin is primarily responsible for cloning and expression of protein probes.	Ms. Su is primarily responsible for cloning and expression of protein probes.
Funding support	School funding	School funding		School funding		

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Nothing to report.

SPECIAL REPORTING REQUIREMENTS

Nothing to report.

APPENDICES

Nothing to report.